

1 **A mechanism for sequence specificity in plant-mediated interactions between**
2 **herbivores**

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13 **Summary**

14 1. Herbivore communities are shaped by indirect plant-mediated interactions whose
15 outcomes are strongly dependent on the sequence of herbivore arrival. However, the
16 mechanisms underlying sequence specificity are poorly understood.

17 2. We examined the mechanisms which govern sequence specific effects between two
18 specialist maize herbivores, the leaf feeder *Spodoptera frugiperda* and the root feeder
19 *Diabrotica virgifera virgifera*. In the field, *S. frugiperda* reduces *D. v. virgifera*
20 abundance, but only when arriving on the plant first.

21 3. In behavioral experiments, *D. v. virgifera* larvae continued feeding on plants that
22 they had infested prior to leaf infestation, but refused to initiate feeding on plants that
23 were infested by *S. frugiperda* prior to their arrival. Changes in root-emitted volatiles
24 were sufficient to elicit this sequence-specific behaviour. Root volatile and headspace
25 mixing experiments showed that early arriving *D. v. virgifera* larvae suppressed *S.*
26 *frugiperda* induced volatile repellents, which led to the maintenance of host
27 attractiveness to *D. v. virgifera*.

28 4. Our study provides a physiological and behavioral mechanism for sequence
29 specificity in plant-mediated interactions and suggests that physiological canalization
30 of behaviorally active metabolites can drive sequence specificity and result in strongly
31 diverging herbivore distribution patterns.

32 **Keywords**

33 above-below ground interactions, *Diabrotica virgifera virgifera*, induced resistance,
34 physiological canalization, plant-herbivore interactions, *Spodoptera frugiperda*,
35 volatile organic compounds, *Zea mays*

36 **Introduction**

37 Interspecific competition influences the structure, function and stability of
 38 natural and agricultural ecosystems (Loreau & de Mazancourt, 2013). For herbivorous
 39 insects, interspecific competition can occur through direct interference or through
 40 plant-mediated, indirect effects (Denno *et al.*, 1995). A growing number of studies
 41 show that plant-mediated, indirect effects are the most common form of interspecific
 42 competition between herbivores (Ohgushi, 2005; Kaplan & Denno, 2007; Xiao *et al.*,
 43 2012; Huang *et al.*, 2013) and that they act as driving forces of herbivore community
 44 composition in nature (Kaplan & Denno, 2007; Poelman & Dicke, 2014; Stam *et al.*,
 45 2014).

46 The outcome of plant-mediated interactions between herbivores is determined by
 47 a number of factors, including the identity of the attacking herbivore, the identity of
 48 the plant and the identity of the responding herbivore (Johnson & Agrawal, 2005;
 49 Wurst & van der Putten, 2007; Xiao *et al.*, 2012; Huang *et al.*, 2014). Recently, the
 50 sequence of arrival was identified as an important factor as well: Depending on which
 51 species arrives first, the effect of one herbivore on the other can change drastically.
 52 Soler *et al.* (2012) for instance observed that *Pieris brassicae* caterpillars grew bigger
 53 when feeding on *Brassica oleracea* plants that were infested by *Brevicoryne*
 54 *brassicae* aphids before the arrival *P. brassicae*, but not if both herbivores attacked
 55 the plant simultaneously. A recent meta-analysis on interactions between leaf and root
 56 feeding herbivores identified the sequence of arrival as a strong predictor for the
 57 directionality of effects for this type of plant-mediated interactions (Johnson *et al.*,
 58 2012).

59 To date, several physiological hypotheses have been proposed that may explain
 60 sequence specificity (Erb *et al.*, 2011a; Stam *et al.*, 2014): Plant-mediated
 61 feedback-loops, overriding induction effects and physiological canalization.
 62 Plant-mediated feedback-loops occur if two herbivores sharing a host plant influence
 63 each other reciprocally (Soler *et al.*, 2012): A first arriving herbivore could then

influence the behavior and damage patterns of a second arriver by inducing physiological changes in the plant, which, by consequence, would change the plant-mediated impact of the second herbivore on the first herbivore and thereby lead to sequence specific patterns. Overriding effects occur if one herbivore elicits a plant response that is much stronger than the response of the other herbivore and thereby determines the resulting interaction (Stam *et al.*, 2014). Physiological canalization is a phenomenon where plant responses are determined by the first arriving herbivore (Viswanathan *et al.*, 2007). By suppressing the response that is normally elicited by a second herbivore, physiological canalization can lead to sequence-specific effects.

Behavioral mechanisms may also lead to sequence specificity (Erb *et al.*, 2011a; Karban, 2011). Asymmetrical host acceptance for instance refers to situations where a herbivore is less likely to start feeding on a new host plant than to continue feeding on a colonized host. This is a common pattern for sedentary herbivores such as miners and gall feeders and may lead to sequence-specific effects by modulating the behavior of a herbivore differently, depending on whether it is arriving on a host plant second or whether it is already established when another herbivore arrives.

Plant physiological and herbivore behavioral mechanisms are not mutually exclusive. Asymmetrical host acceptance for instance may be favored by plant-mediated feedback-loops, overriding effects or physiological canalization: For example, a first arriving herbivore may negatively impact a second herbivore, which may decrease the capability of second herbivore to induce volatile repellents, and in turn render the plant more attractive to first herbivore. Furthermore, a first herbivore may trigger strong physiological changes in the plant which may render it attractive to itself irrespective of the potentially unattractive changes that are induced by a second arriving herbivore. Finally, a first herbivore may change the plant's physiology in a way that makes it unresponsive to the second herbivore, which may lead to the suppression of an otherwise unattractive physiological change. So far, the contributions of the different physiological and behavioral mechanisms and their combinations to sequence specificity have not been tested experimentally. By

93 consequence, the drivers of sequence specificity in indirect, plant-mediated
94 interactions are not well understood.

95 Here, we analyzed potential mechanisms leading to sequence-specificity by
96 studying the effect of attack by the leaf-feeding larvae of *Spodoptera frugiperda* (J.E.
97 Smith) on the root-feeding larvae of *Diabrotica virgifera virgifera* (LeConte) sharing
98 maize (*Zea mays* L.) as a common host plant. Both herbivores occur on cultivated
99 maize and its wild ancestors and cause severe damage in both agricultural and natural
100 systems (Branson & Krysan, 1981; O'Day, 1998). They overlap spatially and
101 temporally in the field, with their sequence of arrival varying considerably with
102 climatic conditions and locations (Branson, 1976; O'Day, 1998). Our previous study
103 within the same system revealed that *S. frugiperda* larvae significantly reduce the
104 number of *D. v. virgifera* larvae feeding on maize roots in the field, but only when *S.*
105 *frugiperda* larvae arrive first (Erb *et al.*, 2011a). Subsequent experiments showed that
106 maize root systems of plants which are attacked by leaf-feeding caterpillars become
107 highly unattractive to *D. v. virgifera* larvae, and that this effect is mediated by long
108 and short distance host acceptance cues (Robert *et al.*, 2012a; Erb *et al.*, 2015; Lu *et*
109 *al.*, 2016). In contrast, *D. v. virgifera* attack renders the plant highly attractive to
110 conspecifics (Robert *et al.*, 2012a) and reprograms the root metabolism to become
111 more suitable for its own development (Robert *et al.*, 2012b). Although *D. v. virgifera*
112 decreases the performance of leaf-feeders on maize under water limiting conditions,
113 which may lead to plant-mediated feedback loops (Erb *et al.*, 2009; Erb *et al.*, 2011b),
114 we found no correlation between the amount of *S. frugiperda* leaf-damage and the
115 reduction of *D. v. virgifera* performance in our previous work (Erb *et al.*, 2011a).

116 Based on the findings above, we hypothesized that asymmetrical host acceptance
117 may contribute to the sequence specific interaction patterns between *D. v. virgifera*
118 and *S. frugiperda*, and that this asymmetrical acceptance behavior may either be the
119 result of overriding effects or physiological canalization. We therefore conducted a
120 series of behavioral experiments to explore the impact of the sequence of arrival on
121 host plant attractiveness and acceptability for *D. v. virgifera* larvae. We then used a

modified two-by-two-arm olfactometer to test the influence of plant volatiles on the sequence-specific behavior of *D. v. virgifera* and to distinguish between overriding effects and physiological canalization. Finally, we analyzed the changes in root volatiles elicited by the different arrival sequences to test for patterns of physiological canalization.

Materials and Methods

Plants and insects

Maize seeds (hybrid Delprim) were obtained from Delley DSP (Delley, Switzerland). They were sown individually in plastic pots (11 cm depth, 4 cm diameter) and placed in a greenhouse (26 ± 2 °C, 14 : 10 h light : dark, 55 % relative humidity). Twelve days later (henceforth called day 0), plants with three fully developed leaves were used for experiments. Eggs of *D. v. virgifera* were obtained from the USDA-ARS (Brookings, SD) and larvae were reared on freshly germinated maize plants until use. *S. frugiperda* eggs were obtained from the University of Neuchatel, (Neuchâtel, Switzerland), and the hatching larvae were reared on soy-wheat germ diet (Bio-Serv, USA) until use.

Plant treatments

To establish different feeding sequences and herbivore combinations, plants were randomly assigned to one of four treatments (Fig. 1a). (1) aboveground herbivory (AG): Twelve second-instar *S. frugiperda* larvae were added to the leaves of each plant at day 2; (2) belowground herbivory (BG): Six second-instar *D. v. virgifera* larvae were added into a hole (9 cm depth, 0.5 cm diameter) in the soil at the base of each plant at day 0; (3) belowground attack followed by aboveground attack (BG>AG): Six second-instar larvae of *D. v. virgifera* were added to each plant at day 0, and twelve second-instar larvae of *S. frugiperda* were added to each plant at day 2; (4) controls without herbivory (C). These treatments simulated a situation where *D. v. virgifera* larvae newly arrive on plants already infested with *S. frugiperda* (AG) or

149 where they can continue feeding on maize plants that are infested by conspecifics
 150 alone (BG) or by conspecifics that arrived prior to the arrival of the leaf feeder
 151 (BG>AG) (Fig. 1a). As *D. v. virgifera* larvae refuse to feed on plants that are
 152 previously attacked by *S. frugiperda* (Robert *et al.*, 2012a; Erb *et al.*, 2015; Lu *et al.*,
 153 2016), an AG>BG treatment was not included in the experimental setups.

154 To prevent above- and belowground herbivores from escaping, the aboveground
 155 parts (leaves of maize plants) were caged with transparent 1.5L plastic bottles with
 156 their bottoms removed that were put upside down on the pots. Belowground parts
 157 (pots) were covered with aluminum foil. All plants were caged the same way
 158 regardless of herbivore treatment. Furthermore, small holes were made in the soil of
 159 each plant regardless of *D. v. virgifera* infestation. Four days after the beginning of
 160 the different treatments (day 4), the plastic bottles and *S. frugiperda* larvae were
 161 removed. Then, the responding *D. v. virgifera* larvae were introduced into the system
 162 as described below (Fig. 1b-e). Timing and herbivore densities were chosen to match
 163 earlier studies and to mimic natural occurrence patterns in the field (Erb *et al.*, 2011a;
 164 Robert *et al.*, 2012a).

165 ***Influence of sequence of arrival on host plant acceptance by D. v. virgifera***

166 In a first set of experiments, we tested the hypothesis that *D. v. virgifera* larvae
 167 may reject roots of plants that are previously infested with *S. frugiperda*, but may
 168 continue to feed on plants on which they were able to establish a suitable feeding
 169 environment prior to the arrival of the leaf-feeder. We conducted experiments using
 170 three different setups, as described below (Fig. 1b-d).

171 First, we tested the behaviour of *D. v. virgifera* using a Petri dish setup which
 172 allowed for direct root contact (Robert *et al.*, 2012c) (Fig. 1b). The root systems of
 173 plants from the different treatment groups were gently washed with tap water. Plants
 174 were then paired in the following combinations: (1) C vs AG; (2) C vs BG; (3) C vs
 175 BG>AG. Root systems of the different plant pairs were placed on a moistened filter
 176 paper in a Petri dish (13.5 cm diameter, 2 cm depth), which had a gap (0.8 cm width,

177 2 cm height) on the side. The stems were laid into the gap, with the leaves remaining
178 outside of the Petri dish. Six second-instar larvae were then added onto the moistened
179 filter paper. The larvae could move and feed freely on the plants within the Petri dish.
180 The Petri dish was covered with aluminum foil to decrease the impact of light on the
181 roots and insects. The position of the larvae was recorded at 0.5h, 1.5h, 3h and 5h.
182 Larvae that remained on the filter paper and did not choose a plant were counted as
183 no-choice. Each treatment combination was repeated 24-36 times.

184 Second, we specifically tested the contribution of volatile cues to the observed
185 behavioral patterns. For this purpose, the same treatment combinations as in the first
186 experiment were offered to *D. v. virgifera* larvae in two-arm olfactometers as
187 described (Robert *et al.*, 2012a) (Fig. 1c). Before the beginning of the treatments,
188 plants were transplanted individually into L-shaped glass pots (11 cm depth, 5 cm
189 diameter) with a horizontal connector at a height of 0.5 cm and filled with moist sand.
190 At day 4, the horizontal connector of each glass pot was attached with one Teflon
191 connector (29 / 32 to 24 / 29 mm) which contained a fine metal screen (2300 mesh;
192 Small Parts Inc.). Then, the two Teflon connectors were linked using a glass tube (24 /
193 29 mm; length 8 cm) with a vertical access port in the middle. To keep the root
194 systems in the dark and to avoid visual cues for the larvae, the entire olfactometer was
195 covered with aluminum foil. Twenty minutes after connecting the different odor
196 sources, six second-instar *D. v. virgifera* larvae were released into the access port of
197 the glass tube. The larvae could move freely in the glass tube, but could not reach the
198 roots of the plants. After 10 min, the olfactometer was disassembled and the number
199 of larvae in each Teflon connector was recorded. Larvae that stayed in the central
200 glass tube after 10 min were recorded as no-choice. For each treatment combination,
201 18 independent replicates were carried out.

202 In a third experiment, we tested whether *D. v. virgifera* larvae are more likely to
203 leave the rhizosphere environment of infested plants, even in the absence of an
204 alternative host. For this purpose, plants were potted and infested in L-shaped glass
205 pots as described above (Fig. 1d). Then, six second-instar larvae were released

206 directly at the entrance of the horizontal access port of each glass pot. The access port
207 of the horizontal connector was not sealed so the larvae could move into the soil and
208 start feeding or try to escape from the plant through the access port. The L-pot was
209 placed in a Petri dish filled with tap water at a height of 0.5 cm to catch escaping *D. v.*
210 *virgifera* larvae without flooding the glass pot. The number of escaped larvae in the
211 trap was recorded over 20 min. For each treatment, 12 replicates were carried out.

212 ***Plant-mediated feedback-loops***

213 To evaluate whether belowground attack by *D. v. virgifera* changes the
214 aboveground damage pattern by *S. frugiperda* larvae under the current experimental
215 conditions, the leaves of plants from the different infestation treatments were
216 collected at day 4, and total leaf area and damaged leaf area were measured for each
217 plant using Digimizer software (MedCalc Software bvba; Mariakerke, Belgium).
218 Eighteen replicates per treatment were carried out.

219 ***Overriding effects***

220 To investigate whether an overriding signal may be responsible for the observed
221 asymmetrical host acceptance of *D. v. virgifera* in the first set of experiments, we
222 developed a two-by-two-arm belowground olfactometer that allowed us to combine
223 the volatile headspaces from two odor sources per arm (Fig. 1e). For this purpose, the
224 setup described above was modified as follows: Two Teflon connectors attached to
225 glass pots were linked using a “Y” glass tube (24 / 29 mm; length 8 cm) at an angle of
226 60°. Then, two “Y” glass tubes were connected to a central glass tube (24 / 29 mm;
227 length 8 cm) with a vertical access port in the middle. This modification enabled us to
228 attach two L-shaped glass pots to each side of the release tubes and to test the
229 preference of *D. v. virgifera* for two combinations of two mixed odor sources. The
230 following treatment combinations were investigated using this setup: C+C vs C+AG;
231 C+C vs C+BG, C+C vs AG+BG. The olfactometer was disassembled and the number
232 of larvae in each “Y” glass tube was recorded after 10 min. We hypothesized that if *D.*

233 *v. virgifera* elicits an overriding signal, the AG+BG arms should be more attractive
234 than the C+C arm. Eighteen replicates were performed for each treatment
235 combination.

236 ***Physiological canalization***

237 To evaluate whether *D. v. virgifera* attack canalizes the root volatile response in
238 a way that suppresses responsiveness to *S. frugiperda* infestation, we collected and
239 analyzed root volatile profiles using solid-phase micro-extraction-gas
240 chromatography-mass spectrometry (SPME-GC-MS). Plants were treated as
241 described above (Fig. 1a). Crown and primary roots were then washed with tap water
242 and frozen in liquid nitrogen. Twelve plants per treatment were harvested, and the
243 roots of two plants were pooled for analysis, resulting in six biological replicates. The
244 crown and primary roots of each replicate were ground into a fine powder, and 50 mg
245 of each root type were placed in a 10 ml glass vial and sealed using Teflon tape
246 (Polytetrafluoroethylene). An SPME fiber (100 μ m polydimethylsiloxane coating,
247 Supelco, USA) was then inserted into the vial for 60 min at 50°C. The incubated
248 fibers were then immediately analyzed by GC-MS (Agilent 7820A GC interfaced
249 with an Agilent 5977E MSD) following previously established protocols with a few
250 modifications (Erb *et al.*, 2011c). Briefly, the fiber was inserted into the injector port
251 at 250 °C and desorbed for 2 min. After fiber insertion, the column temperature was
252 maintained at 60 °C for 1 min and then increased to 250 °C at 5 °C min⁻¹ followed by
253 a final stage of 4 min at 250 °C. The overall analysis time for each sample, including
254 oven cooling, was 45 min. Furthermore, to eliminate the impact of background peaks,
255 three glass vials without any plant material (blanks) were run using the same protocol
256 as described above. The resulting GC-MS chromatograms were processed with
257 Progenesis Q1 (informatics package from Waters, MA, USA) using default settings for
258 spectral alignment and peak picking. From the resulting matrix, all features which
259 were presented in more than one blank were removed, resulting in 232 features.
260 Features were assigned to individual compounds by retention time and peak shape
261 matching and identified using the NIST search 2.2 Mass Spectral Library as well as

262 retention time and spectral comparison with pure compounds.

263 ***Data analysis***

264 To examine host acceptance of *D. v. virgifera* in a Petri dish experiment, the
265 number of larvae found on different herbivory treatment groups was analyzed using a
266 Wald test applied on a Generalized Linear Mixed Model (GLMM) with a Poisson
267 distribution. We considered plant treatment as a fixed factor, time as covariate and the
268 replicate as a random factor. Each plant combination (C vs AG, C vs BG and C vs
269 BG>AG) was analyzed separately. Then, to compare the preference of *D. v. virgifera*
270 between the different treatment groups, the number of larvae on infested plants (AG,
271 BG and BG>AG) was analyzed using a likelihood ratio test applied on a Generalized
272 Linear Model (GLM) with a Poisson distribution. The models included herbivory as
273 fixed factor and time as a covariate. The preference of *D. v. virgifera* larvae in the
274 olfactometer experiments and the number of escaped larvae in escape experiment
275 were analyzed as described above. To examine whether belowground attack by *D. v.*
276 *virgifera* larvae changes the aboveground damage pattern by *S. frugiperda* larvae, the
277 relative and absolute leaf damage of *S. frugiperda* larvae was analyzed using
278 independent sample *t*-tests (BG vs BG>AG). The absolute leaf damage was estimated
279 by the sum of leaf damaged area for each plant and the relative leaf damage was
280 calculated as the sum of leaf damaged area / the sum of total leaf area \times 100 for each
281 plant. To examine the overall differences in volatile profiles, the relative abundance of
282 the detected features were subjected to redundancy analysis (RDA) using the different
283 treatments as a unique explanatory variable. Monte Carlo tests with 999 permutations
284 were then used to test for significant differences between treatments. For more
285 detailed, compound specific analyses, the different features were assigned to
286 individual compounds, and the relative abundance of the individual compounds,
287 which corresponds to the sum of the signal intensities of the individual features, were
288 analyzed by one-way ANOVAs followed by least square mean *post-hoc* tests for
289 pairwise comparisons, including false discovery rate (FDR) corrections (Benjamini &
290 Hochberg, 1995). All analyses were conducted using R 3.2.0 (R Foundation for

291 Statistical Computing, Vienna, Austria) with “car”, “lme4”, “lsmeans”, “vegan” and
 292 “RVAideMemoire” packages (Fox & Weisberg, 2011; Bates *et al.*, 2015; Hervé, 2016;
 293 Lenth, 2016; Oksanen *et al.*, 2016).

294 Results

295 *D. v. virgifera* rejects *S. frugiperda* infested plants only when arriving second

296 In the Petri dish experiment, *D. v. virgifera* larvae strongly preferred the roots of
 297 control plants when offered uninfested vs. leaf-infested plants ($X^2 = 30.753$, $P < 0.001$,
 298 Fig. 2a). By contrast, the larvae showed a strong preference for roots that were
 299 previously infested with *D. v. virgifera* larvae over controls ($X^2 = 69.919$, $P < 0.001$,
 300 Fig. 2b). Roots which were infested with *D. v. virgifera* two days before the onset of *S.*
 301 *frugiperda* attack remained highly attractive ($X^2 = 21.734$, $P < 0.001$, Fig. 2c). The
 302 number of responding *D. v. virgifera* larvae increased with experimental time (C vs
 303 AG, $X^2 = 5.698$, $P = 0.017$; C vs BG, $X^2 = 20.033$, $P < 0.001$; C vs BG>AG, $X^2 =$
 304 35.964 , $P < 0.001$; Fig. 2). At the end of the experiment, 65%, 67% and 70% of *D. v.*
 305 *virgifera* larvae made a choice in C vs AG, C vs BG and C vs BG>AG, respectively.
 306 No significant interactive effects between time and treatments were found (C vs AG,
 307 $X^2 = 3.515$, $P = 0.061$; C vs BG, $X^2 = 0.135$, $P = 0.713$; C vs BG>AG, $X^2 = 1.342$, $P =$
 308 0.247). Overall, more *D. v. virgifera* larvae fed on BG and BG>AG roots than on AG
 309 roots ($X^2 = 38.558$, $P < 0.001$, Fig. 2). No difference was found between the
 310 preference of *D. v. virgifera* for BG and BG>AG roots ($P = 0.064$, Fig. 2).

311 In the two-arm olfactometer experiment, similar preference patterns were
 312 observed. *D. v. virgifera* larvae showed a strong preference for control plants over *S.*
 313 *frugiperda* infested plants ($X^2 = 8.111$, $P < 0.01$, Fig. 3). By contrast, the larvae
 314 preferred plants that were previously infested with conspecifics over controls ($X^2 =$
 315 34.177 , $P < 0.001$, Fig. 3). Plants were infested with *D. v. virgifera* prior to *S.*
 316 *frugiperda* infestation remained highly attractive ($X^2 = 16.849$, $P < 0.001$, Fig. 3). In
 317 this experiment, all larvae made a choice within 10 min. Together, the larvae were

more attracted to the roots that had been infested by conspecifics alone and conspecifics that had arrived prior to the arrival of the *S. frugiperda*, while were less attracted to the roots that had been infested by *S. frugiperda* alone ($\chi^2 = 20.396$, $P < 0.001$, Fig. 3). Again, BG and AG>BG treatments were not significantly different from each other ($P = 0.389$, Fig. 3).

When offered a single host plant, the number of escaping *D. v. virgifera* larvae differed significantly between treatments ($\chi^2 = 32.112$, $P < 0.001$, Fig. 4). When offered a *S. frugiperda* infested plant, 50% of the larvae escaped from the rhizosphere within 20 min (Fig. 4). By contrast, less than 18% of the larvae left the soil of control plants or plants that were previously infested with conspecifics (Fig. 4). A similar percentage of larvae chose to remain in the rhizosphere of plants that were infested with *D. v. virgifera* prior to *S. frugiperda* attack (Fig. 4).

Plant-mediated feedback-loops are unlikely to explain D. v. virgifera behavior

There was no significant difference in relative ($t = 0.055$, $P = 0.957$) or absolute ($t = 1.236$, $P = 0.225$) damaged leaf area between plants from that were infested with *D. v. virgifera* or root herbivore free (Fig. S1). These results suggest that the interaction between *D. v. virgifera* and *S. frugiperda* is highly asymmetrical and that plant-mediated feedback-loops are unlikely to play a major role in determining sequence specific responses of *D. v. virgifera*.

D. v. virgifera does not produce an overriding attractive signal

Similarly to the two-arm olfactometer experiment, *D. v. virgifera* larvae significantly preferred to move to the side of the olfactometer containing two control plants rather than the arm leading to a control plant and an *S. frugiperda* infested plant ($\chi^2 = 15.446$, $P < 0.001$, Fig. 5). The opposite was true for a combination of a control plant with a *D. v. virgifera* infested plant, which was attractive to the root feeder ($\chi^2 = 8.111$, $P < 0.01$, Fig. 5). In contrast to the attractiveness of BG>AG plants observed in the two-arm olfactometer experiment however (Fig. 3), the mixed rhizosphere

345 volatiles from an *S. frugiperda* and a *D. v. virgifera* infested plant were highly
 346 unattractive, and significantly more larvae moved to the control side ($\chi^2 = 10.333$, $P <$
 347 0.01, Fig. 5) than to the AG+BG side. All larvae made a choice within the first 10 min.
 348 Overall, the presence of plants that were infested by *S. frugiperda* significantly
 349 repelled *D. v. virgifera* ($\chi^2 = 15.915$, $P < 0.001$, Fig. 5). This experiment falsifies the
 350 hypothesis that *D. v. virgifera* triggers an overriding attractant.

351 ***D. v. virgifera* feeding suppresses *S. frugiperda* induced root volatiles**

352 In total, we detected 232 volatile features in the GC-MS chromatograms.
 353 Redundancy analysis revealed that *S. frugiperda* and *D. v. virgifera* attack induced
 354 different volatile blends compared to control plants and compared to each other (AG
 355 vs C: $P = 0.008$; BG vs C: $P = 0.008$; BG>AG vs C: $P = 0.008$, Fig. 6). The volatile
 356 profiles of plants that were induced by *D. v. virgifera* prior to *S. frugiperda* attack
 357 were indistinguishable from plants that were infested with *D. v. virgifera* alone
 358 (BG>AG vs BG: $P = 0.642$, Fig. 6), but both of them were significantly different from
 359 plants that were infested with *S. frugiperda* alone (BG vs AG: $P = 0.008$; BG>AG vs
 360 AG: $P = 0.008$, Fig. 6). Analysis of variance revealed twelve volatile compounds
 361 whose abundance differed significantly between treatments (Fig. 7). Pairwise
 362 comparisons showed that four of these volatiles were significantly induced by *D. v.*
 363 *virgifera* infestation alone (Fig. 7a-d) and two of them were significantly induced by *S.*
 364 *frugiperda* attack alone (Fig. 7k-l). We found no significant effect of later *S.*
 365 *frugiperda* attack on *D. v. virgifera* induced volatile emissions (Fig. 7). However, the
 366 induction of the *S. frugiperda* induced volatiles was suppressed by early *D. v.*
 367 *virgifera* infestation (Fig. 7l). This result demonstrates that *D. v. virgifera* canalizes
 368 the root volatile production and renders roots unresponsive to leaf-attack by *S.*
 369 *frugiperda*.

370 **Discussion**

371 The sequence of arrival is increasingly recognized as an important determinant

372 of plant-mediated indirect interactions between herbivores (Viswanathan *et al.*, 2005;
373 Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Erb *et al.*, 2011a; Soler *et al.*, 2012;
374 Wang *et al.*, 2014). However, the mechanisms leading to sequence specificity are not
375 well understood. The goal of the present study was to identify the (mutually
376 non-exclusive) behavioral and physiological mechanisms that may contribute to
377 sequence specific effects. Our experiments show that leaf attack by *S. frugiperda*
378 strongly reduces the attractiveness of roots for *D. v. virgifera* through changes in
379 volatile cues. However, prior *D. v. virgifera* attack suppresses these changes and
380 thereby maintains the attractiveness of the plants to *D. v. virgifera* larvae. This form of
381 asymmetrical host acceptance behavior explains why *S. frugiperda* reduces the
382 abundance and damage by *D. virgifera* in the field only when arriving first on the
383 plant (Erb *et al.*, 2011a).

384 Several non-exclusive physiological mechanisms may explain why *D. v.*
385 *virgifera* is repelled by *S. frugiperda* attacked plants only when arriving second. It is
386 for instance possible that early arriving *D. v. virgifera* larvae change the behavior and
387 induction pattern of *S. frugiperda*. However, we found no evidence for the presence of
388 resistance feedback loops in our system: *S. frugiperda* damage remained unchanged
389 by *D. v. virgifera* attack. Earlier studies demonstrated that *D. v. virgifera* root attack
390 increases leaf resistance via ABA signalling under drought conditions; when plants
391 are well watered, no negative effects of *D. v. virgifera* on *Spodoptera littoralis* growth
392 were observed any more (Erb *et al.*, 2011b). The maize seedlings in our experiments
393 were supplied with sufficient soil moisture, which likely prevented potential feedback
394 loops from occurring. Another explanation for the observed behavioural patterns is
395 that *D. v. virgifera* may induce changes that strongly increase the attractiveness of the
396 roots and override any negative changes that are later induced by *S. frugiperda*. By
397 mixing volatiles from different plants, we tested this hypothesis on a behavioral level.
398 Surprisingly, we found that *D. v. virgifera* rejected the volatile mix from a
399 combination of plants that had been infested by *D. v. virgifera* and *S. frugiperda*
400 separately. This is in stark contrast with the strong attractiveness of plants that were

infested with *D. v. virgifera* and *S. frugiperda* sequentially and strongly suggests that *D. v. virgifera* does not produce an overriding attractive signal.

On the other hand, our GC-MS analyses provide clear evidence that *D. v. virgifera* canalizes the plant's root volatile response. Maize roots responded strongly to *D. v. virgifera* attack and produced higher amounts of several volatiles, including several products of the terpene synthase TPS23 which are strongly induced by *D. v. virgifera* (Köllner *et al.*, 2008; Hiltbold *et al.*, 2011) and attract the root feeder (Robert *et al.*, 2012a). These responses were not altered by later *S. frugiperda* attack. By contrast, *S. frugiperda* attack induced a different set of compounds in the roots, including a yet unidentified nitrophenol, and this induction was fully suppressed by prior *D. v. virgifera* attack. These results demonstrate that early arriving *D. v. virgifera* canalizes the root metabolism in a way that makes it unresponsive to *S. frugiperda* attack. Canalization of plant responses by herbivores has been proposed to occur in a number of plant-herbivore interactions (Thaler *et al.*, 2002; Viswanathan *et al.*, 2005; Utsumi *et al.*, 2010). For example, Viswanathan *et al.* (2007) found that tortoise beetle attack after flea beetle attack of *Solanum dulcamara* did not alter the induced resistance elicited by the flea beetles. By contrast, tortoise beetle attack before flea beetle attack resulted in the disappearance of induced resistance. One possible explanation of canalization is negative cross-talk between signaling pathways that inducing one pathway may attenuate or repress other pathways (Koornneef & Pieterse, 2008; Erb *et al.*, 2012). Furthermore, priority in occupying a plant resource may also result in physiological canalization, as resources invested into an initial induced response may be not available for investment into later induced responses (Stam *et al.*, 2014). In combination with the behavioral experiments, these results suggest that the asymmetrical host acceptance behavior of *D. v. virgifera* is caused by physiological canalization.

In a previous study, we found that leaf attack by *S. littoralis* leads to a slight decrease in root ethylene production, and that adding ethylene back to the root system restores the attractiveness of the roots to *D. v. virgifera* (Robert *et al.*, 2012a). Many

herbivores increase local ethylene emissions of their host plants (Winz & Baldwin, 2001; von Dahl & Baldwin, 2007; Schäfer *et al.*, 2011), and it is therefore possible that *D. v. virgifera* attack resulted in the reversal or canalization of the ethylene response of the roots. Unfortunately, ethylene emissions could not be measured in the current series of experiments. However, the presented findings suggest that *S. frugiperda* attack also triggers the release of repellent volatiles which are suppressed by *D. v. virgifera*. The escape experiment in particular shows that *D. v. virgifera* systematically moves away from leaf-infested plants, and it seems unlikely that a reduction in ethylene levels alone can account for this result. Furthermore, the volatile mixing experiment suggests that the volatile blend of the roots of an *S. frugiperda* attacked plant overrides the attractive signal from a *D. v. virgifera* infested root system.

In our GC-MS chromatograms, we found several volatiles which increased in the roots of *S. frugiperda* attacked plants. Elucidating their structure and bioactivity is an exciting prospect of this work. A recent paper identified methyl antranilate as a repellent for neonate *D. v. virgifera* larvae (Bernklau *et al.*, 2016). Although methyl antranilate was not among the *S. frugiperda* induced root volatiles, it provides an interesting starting point to identify the volatiles which render *S. frugiperda* attacked plants repellent to *D. v. virgifera* larvae. One aspect that should be kept in mind is that root volatiles were measured by grinding root material and sampling the headspace of the ground samples by SPME. The advantages of this technique are its sensitivity and robustness. Its disadvantage is that it may result in the detection of volatile compounds which are not actually released into the rhizosphere by intact roots. Future experiments should therefore include *in vivo* sampling techniques to confirm the release of the newly detected volatiles into the rhizosphere (Ali *et al.*, 2010; Hiltbold *et al.*, 2011).

Host location and acceptance by herbivores are key processes in plant-herbivore interactions. Our results show that physiological canalization can have a strong, sequence-specific impact on host acceptance by herbivores, which may result in

459 strongly diverging herbivore damage and distribution patterns in the field. Our
460 previous work shows that the repellent effect of leaf infestation on root herbivores is
461 highly conserved across herbivore species and maize genotypes (Lu *et al.*, 2016).
462 Whether similar effects also occur in other plant species remains to be elucidated.
463 Understanding the mechanisms which govern sequence specificity will allow for the
464 integration of this phenomenon into current theory on plant-mediated interactions and
465 will facilitate future efforts to develop predictive ecophysiological models of
466 multi-herbivore dynamics on shared host plants.

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473 L.H., Z.B. and C.A.M.R. carried out experiments. W.H., M.R.H. and M.E. analyzed
474 data. M.E. and W.H. wrote the manuscript.

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619 Figure legends

620 **Fig. 1** Overview of the experimental design and setups used in this study. (a)
 621 Experimental treatments (infestation histories). To establish different sequences of
 622 arrival, second instar *S. frugiperda* larvae were added to the leaves, and second instar
 623 *D. v. virgifera* larvae were added to the roots of maize plants in different
 624 combinations. After 4 days of herbivore infestation, plants with different infestation
 625 histories were offered to *D. v. virgifera* larvae in choice and no-choice experiments
 626 and chemical analysis. AG: aboveground *S. frugiperda* larvae infestation, BG:
 627 belowground *D. v. virgifera* larvae infestation, BG>AG: belowground infestation
 628 followed by aboveground infestation, C: control without herbivory. (b) Larval
 629 preference was measured by laying out the root systems of two plant on moist filter
 630 paper in large petri dishes. (c) Volatile-mediated larval preference was measured using
 631 a two arm belowground olfactometer. (d) Larval escape patterns were measured using
 632 a single L-shaped glass pot and a water-filled petri dish to collect the escaping larvae.
 633 (e) Volatile mixing experiments were conducted using a two arm belowground
 634 olfactometer with two volatile sources attached to each arm of the central chamber.
 635 For more details on the different treatments and setups, refer to the materials and
 636 methods section.

637 **Fig. 2** Sequence of arrival determines root attractiveness to *D. v. virgifera*. The
 638 number of *D. v. virgifera* larvae on the roots of plants with different infestation
 639 histories was measured in Petri dish experiment. (a) *D. v. virgifera* choice between C
 640 and AG plants (n = 24). (b) *D. v. virgifera* choice between C and BG plants (n = 36).
 641 (c) *D. v. virgifera* choice between C and BG>AG plants (n = 36). AG: aboveground *S.*
 642 *frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae infestation,
 643 BG>AG: belowground infestation followed by aboveground infestation, C: control
 644 without herbivory. Values correspond to means \pm 1 s.e. Asterisks indicate a significant
 645 difference in preference within each combination and time point (n.s. , non significant;
 646 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, GLMM). Differences in preference patterns
 647 between treatment combinations are depicted by dashed lines and asterisks on the

648 right of the graph (n.s., non significant; *** $P < 0.001$, GLM).

649 **Fig. 3** Volatile cues contribute to sequence-specific preference patterns of *D. v.*
 650 *virgifera*. The number of *D. v. virgifera* larvae attracted to root volatiles of plants with
 651 different infestation histories was measured in two-arm olfactometers experiment. AG:
 652 aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae
 653 infestation, BG>AG: belowground infestation followed by aboveground infestation,
 654 C: control without herbivory. Values are means \pm 1 s.e. (n = 18). Asterisks indicate a
 655 significant preference within each treatment combination (** $P < 0.01$, *** $P < 0.001$;
 656 GLMM). Different letters indicate significant differences between treatment
 657 combinations ($P < 0.05$, GLM).

658 **Fig. 4** Stay-or-leave patterns of *D. v. virgifera* are determined by the sequence of
 659 arrival. The number of *D. v. virgifera* larvae leaving from the rhizosphere of plants
 660 with different infestation histories was measured in escaping experiment. AG:
 661 aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae
 662 infestation, BG>AG: belowground infestation followed by aboveground infestation,
 663 C: control without herbivory. Values are means \pm 1 s.e. (n = 12). Different letters
 664 indicate significant differences between treatments ($P < 0.05$, GLM).

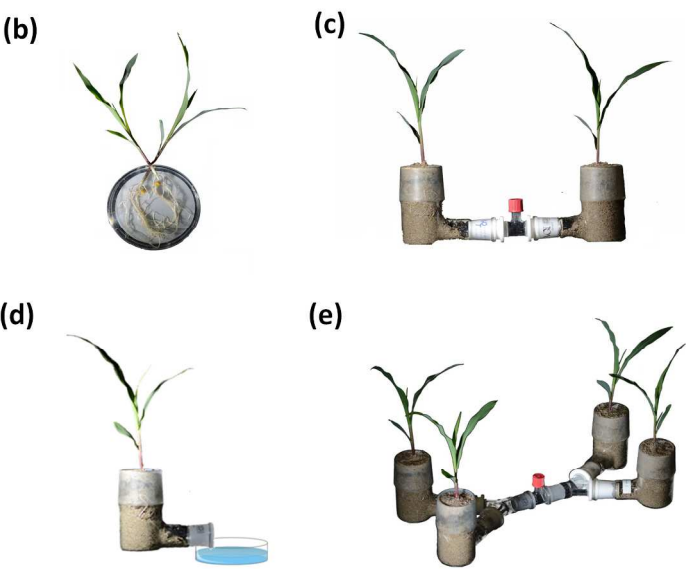
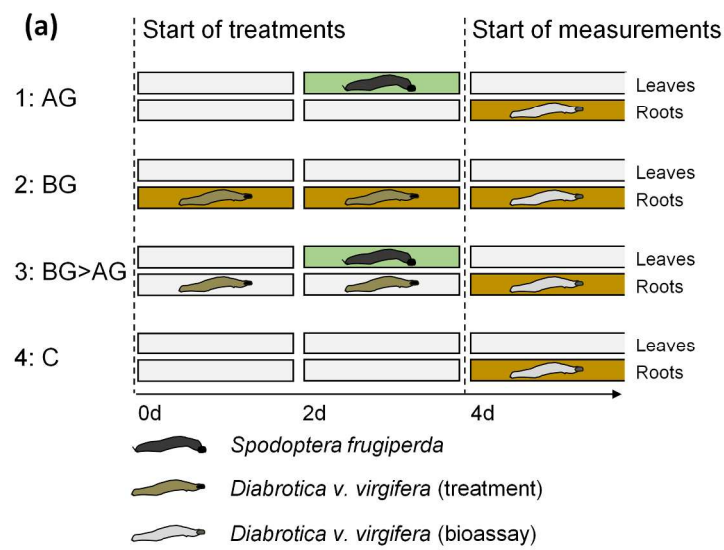
665 **Fig. 5** Acceptances of *D. v. virgifera* are determined by the additive changes in root
 666 volatiles. The number of *D. v. virgifera* larvae attracted by mixed root volatiles from
 667 plants with different infestation histories were measured in volatile-mixing
 668 experiment, with each arm containing two different volatile sources. AG:
 669 aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae
 670 infestation, C: control without herbivory. Values are means \pm 1 s.e. (n = 18). Asterisks
 671 indicate a significant preference within choice combinations (**, $P < 0.01$; *** $P <$
 672 0.001 ; GLMM). Different letters indicate differences in preference patterns between
 673 treatments ($P < 0.05$, GLM).

674 **Fig. 6** Infestation by *D. v. virgifera* canalizes the volatile response of maize roots. The

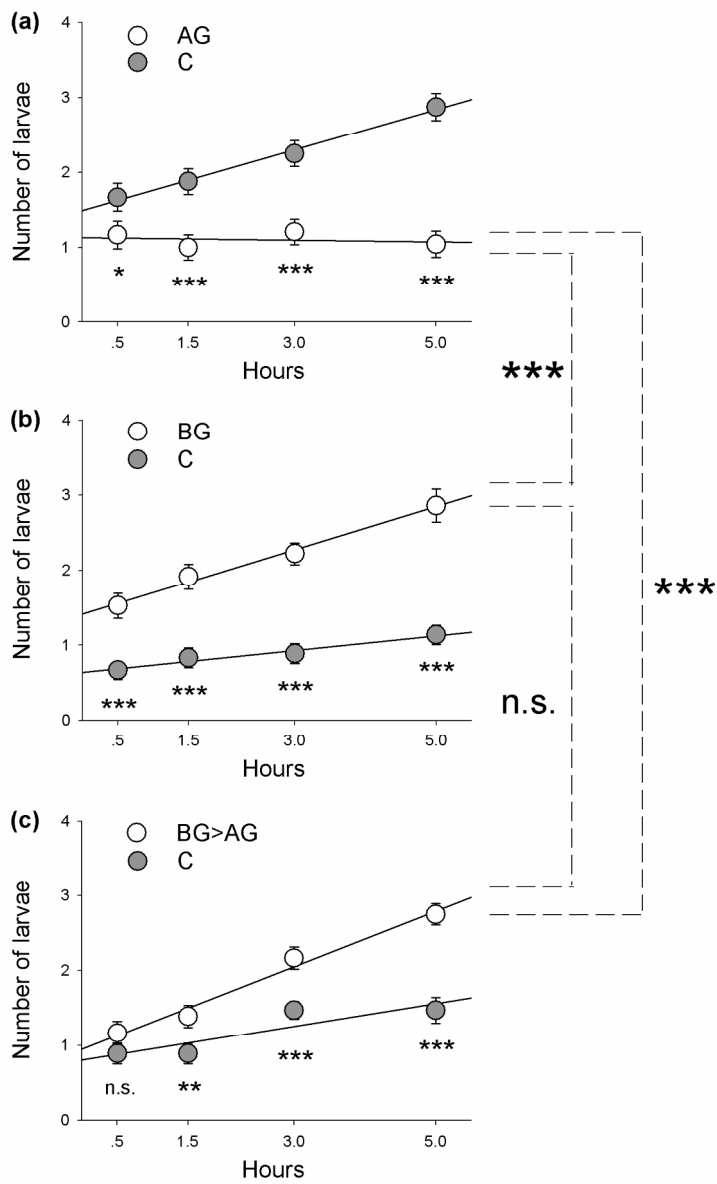
675 results of a redundancy analysis (RDA) of the root volatile responses to different
 676 sequences of *D. v. virgifera* and *S. frugiperda* feeding are shown. The first two axes
 677 explained 53.86% and 24.36% of the total variation. AG: aboveground *S. frugiperda*
 678 larvae infestation, BG: belowground *D. v. virgifera* larvae infestation, BG>AG:
 679 belowground infestation followed by aboveground infestation, C: control without
 680 herbivory. Data points represent individual replicates (n = 6).

681 **Fig. 7** *D. v. virgifera* suppresses *S. frugiperda*-induced root volatiles. The relative
 682 abundance of root volatile in four treatments were measured using solid phase micro
 683 extraction (SPME) in combination with gas chromatography and mass spectrometry
 684 (GC-MS). (a) *E*- β -Caryophyllene (17.33min, 189.1726 m/z), (b) Humulene (18.17min,
 685 204.1966 m/z), (c) Unknown (19.30min, 503.6733 m/z), (d) Unidentified Carboxylic
 686 acid (10.07min, 123.0129 m/z), (e) Unknown (19.07min, 173.0813 m/z), (f)
 687 Caryophyllene oxide (21.27min, 161.1235 m/z), (g) Unknown (19.15min, 106.0578
 688 m/z), (h) Ethanol acetate (15.99min, 204.1814 m/z), (i) Unknown (17.07min,
 689 161.0902 m/z), (j) Unknown (25.05min, 180.0533 m/z), (k) Unknown (12.71min,
 690 138.0904 m/z) and (l) Unidentified nitrophenol (17.67min, 139.0342 m/z). AG:
 691 aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae
 692 infestation, BG>AG: belowground infestation followed by aboveground infestation,
 693 C: control without herbivory. Values are means \pm 1 s.e. (n = 6). Different letters
 694 indicate differences in relative abundance among treatments ($P < 0.05$, LM).

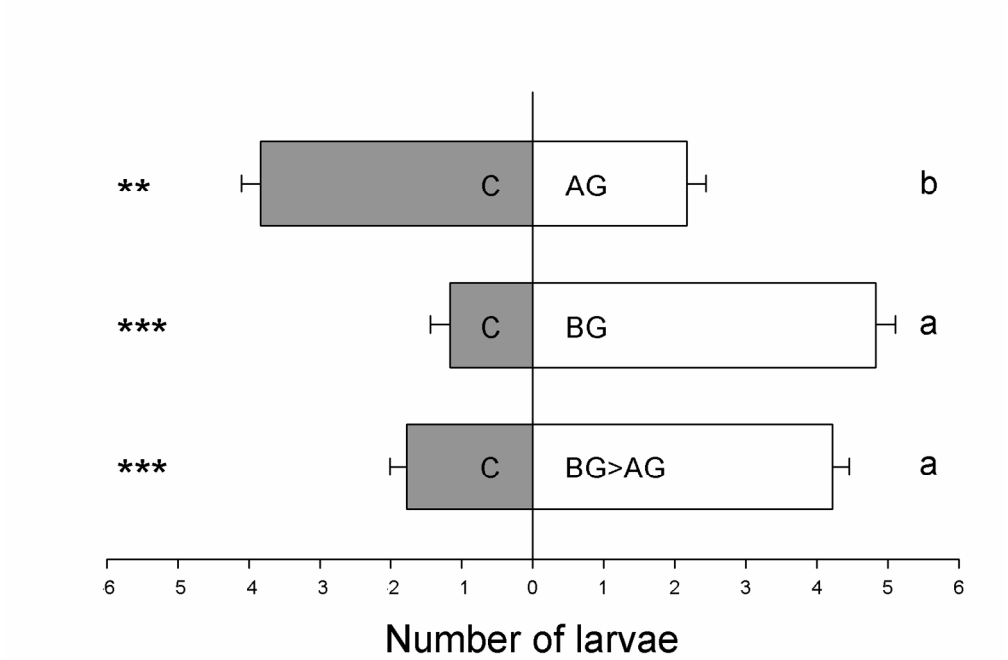
695 **Fig. S1.** Infestation by *D. v. virgifera* does not change aboveground damage by *S.*
 696 *frugiperda* larvae. Relative and absolute leaf damage caused by *S. frugiperda* on
 697 plants with and without previous infestation by *D. v. virgifera* is shown. AG:
 698 aboveground *S. frugiperda* larvae infestation, BG>AG: belowground infestation
 699 followed by aboveground infestation. Values are means \pm 1 s.e. (n = 18).



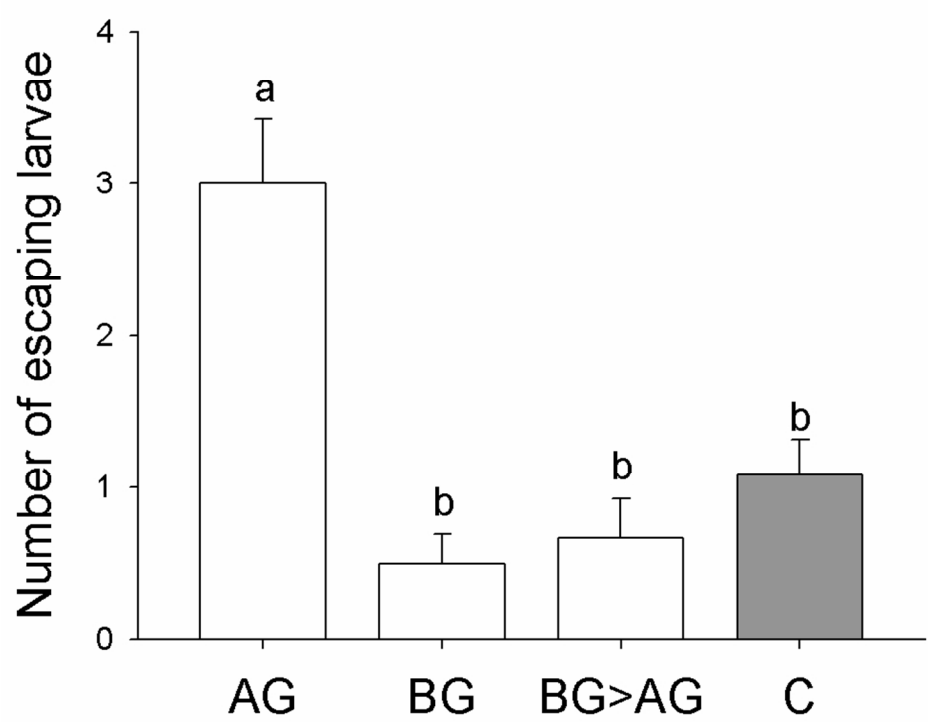
Overview of the experimental design and setups used in this study
Fig. 1
190x274mm (284 x 284 DPI)



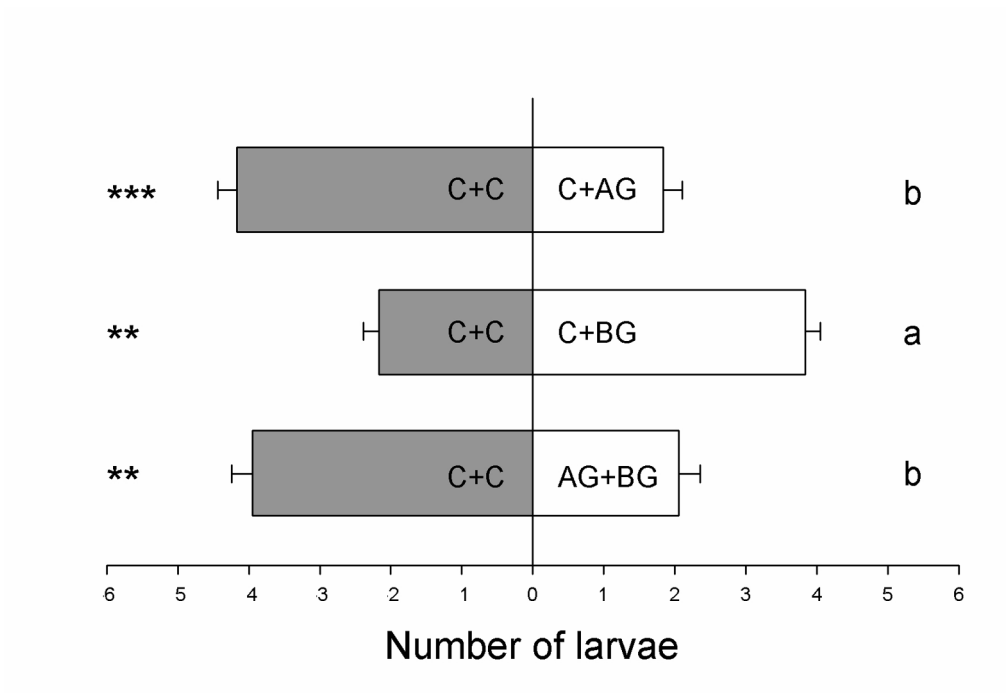
Sequence of arrival determines root attractiveness to *D. v. virgifera*
Fig. 2
128x216mm (300 x 300 DPI)



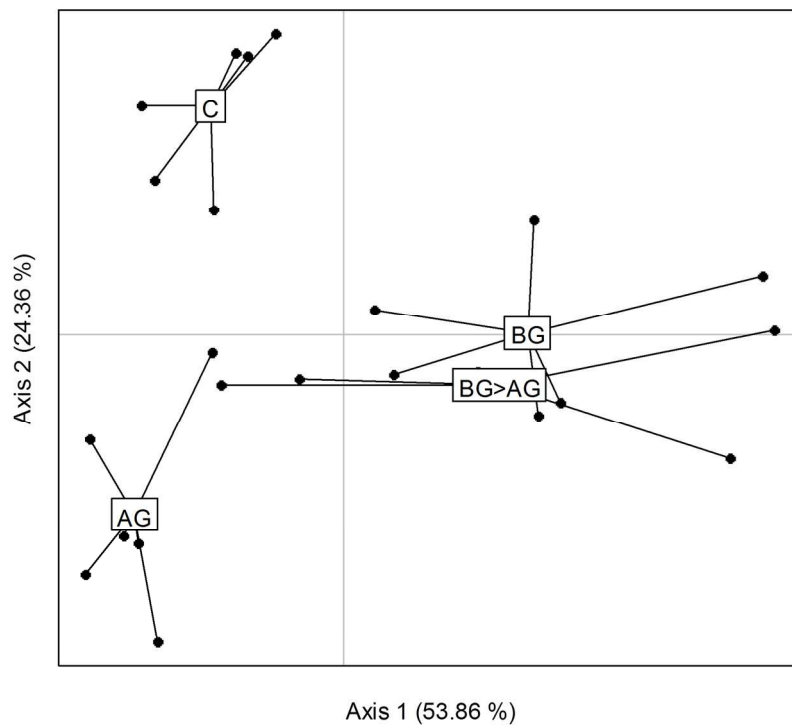
Volatile cues contribute to sequence-specific preference patterns of *D. v. virgifera*
Fig. 3
146x97mm (300 x 300 DPI)



Stay-or-leave patterns of *D. v. virgifera* are determined by the sequence of arrival
Fig. 4
88x78mm (300 x 300 DPI)



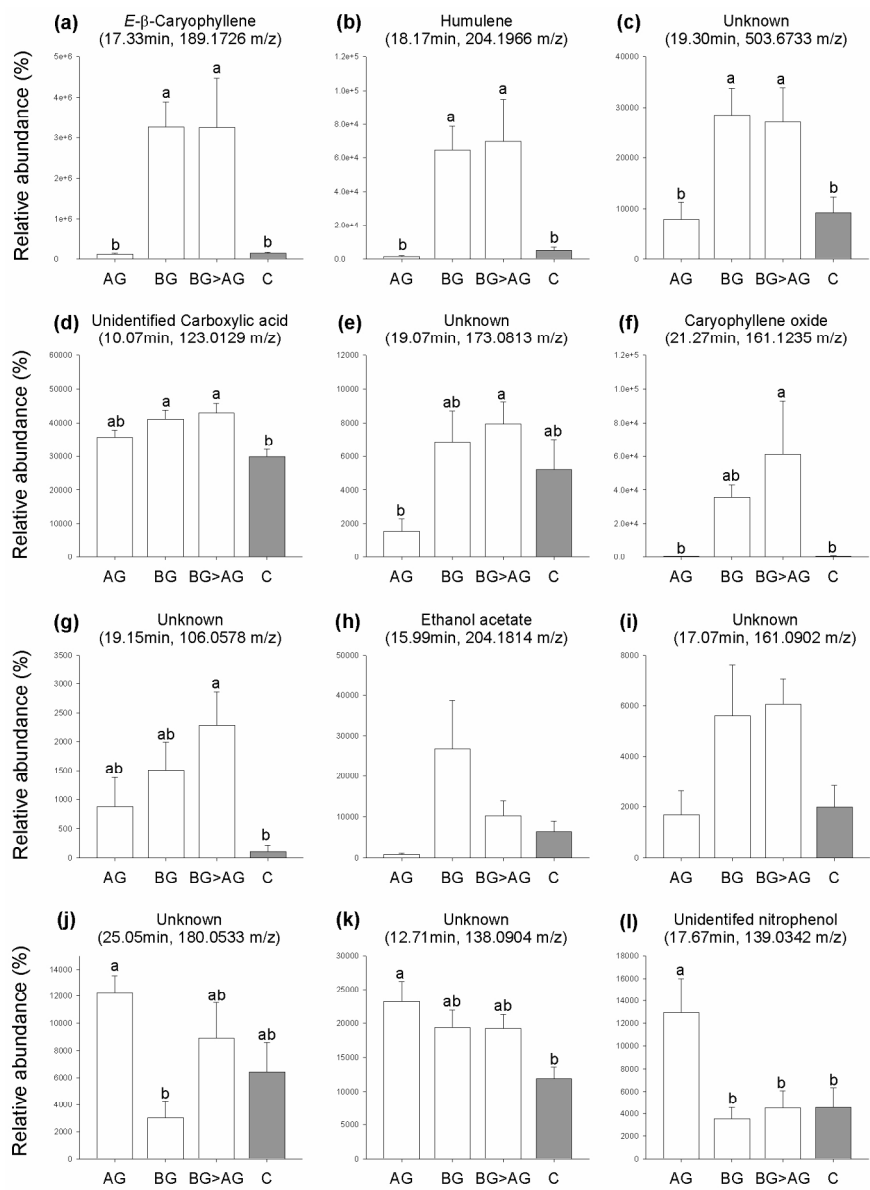
Accptances of *D. v. virgifera* are determined by the additive changes in root volatiles
Fig. 5
146x101mm (300 x 300 DPI)



Infestation by *D. v. virgifera* canalizes the volatile response of maize roots

Fig. 6

190x142mm (300 x 300 DPI)



D. v. virgifera suppresses S. frugiperda-induced root volatiles
Fig. 7
160x213mm (300 x 300 DPI)